p185^{neu} is expressed in yolk sac during rat postimplantation development

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ABSTRACT

We have shown that the *neu* oncogene product (p185^{neu}) is not present in the rat embryo before organogenesis. However, coincident with the onset of organogenesis, p185^{neu} was detected in neural and connective tissue as well as in the secretory epithelium as was described by Kokai et al. (1987). In addition, p185^{neu} is also expressed in the rat visceral yolk sac (VYS) endodermal cells but not in the mesenchymal and mesothelial layers of the same structure nor in the amnion. The first detectable sign of p185^{neu} expression in VYS was found at d 11 of gestation and the levels of protein increased towards the end of pregnancy. In the yolk sac carcinoma (YSC), which is considered to be the malignant counterpart of the rat yolk sac, p185^{neu} was observed only within columnar epithelial cells (the visceral component of the neoplasm) while parietal endoderm-like cells were devoid of detectable protein. From d 9 of pregnancy up to delivery some of the trophoblastic giant cells also showed a faint to moderate immunoreactivity. Results are presented which would indicate a possible role of p185^{neu} in rat embryogenesis.

Key words: Rat embryo; p185^{neu} oncogene.

INTRODUCTION

The *neu* gene (*c-erbB-2/HER-2*) was first identified in a chemically induced rat neuroblastoma (Schechter et al. 1984). This gene codes for a 185 kDa trans-membrane glycoprotein (p185^{neu}) with tyrosine kinase activity and is structurally similar to (but distinct from) the epidermal growth factor receptor (EGFR) (Coussens et al. 1985). The *neu* product, p185^{neu} has been reported to be amplified and/or overexpressed, predominantly in carcinomas of glandular epithelial origin and in derived cell lines (Yokota et al. 1986; Slamon et al. 1987; Yonemura et al. 1991; Gusterson, 1992; Kapitanovic et al. 1992). No ligand for p185^{neu} has been definitively characterised although several peptides have been shown to bind to this 'receptor' (Lupu et al. 1990; Yarden & Peles 1991). Probably the best ligand candidate for p185$^{erbB-2/neu}$ is the 45 kDa protein heregulin-a (HRG-a) which was purified from the conditioned medium of a human breast tumour cell line (Holmes et al. 1992). Heregulin-a is a member of the EGF family and specifically induces phosphorylation of human p185$^{erbB-2/neu}$.

The visceral wall of the yolk sac (VYS) is a particularly important placental organ in rodents because it is the primary mediator of exchange between embryo and mother during early organogenesis. It consists of 3 layers: visceral endoderm cells, a mesenchymal component and a mesothelial cell layer. Endodermal cells are engaged in adsorptive pinocytosis, which leads to the preferential uptake of macromolecules. These macromolecules are then degraded in lysosomes and transported into the blood vessels located within the mesenchymal component and used by the embryo as an energy source for growth and development (Jollie, 1990). Therefore, the endodermal cell layer acts functionally as a true glandular tissue by processing specific molecules and then exporting them from cells to be used elsewhere in the developing embryo.

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It has previously been shown that during normal rat development the *neu* oncogene is expressed in the nervous system, connective tissue, and secretory epithelium, but not in lymphoid tissue (Kokai et al. 1987). However, that report did not include expression data concerning embryonic stages before organogenesis, nor the possible distribution of the *neu* protein in the embryonic membranes (yolk sac and amnion) and placenta. We therefore investigated the presence of p185^{neu} in these tissues. Furthermore, we investigated the presence of p185^{neu} in the malignant counterpart of the embryonic membrane (yolk sac carcinoma).

MATERIALS AND METHODS

Embryos

The inbred Fisher strain of the albino rat was used in the experiment. Males and females were caged together overnight and pregnancy was confirmed the following morning by the presence of sperm cells in the vaginal smear. This day was considered as d 1 of gestation. Pregnant animals were killed on the desired days by means of ether vapour overdose. Embryos were harvested from as early as the primitive streak stage (8 d embryo, d 9 of gestation) up to as late as a few hours before delivery (d 21 of gestation). At least 3 pregnant females contributed embryos to each developmental stage studied. A total of 155 embryos were included in this study.

On d 9 and 10 of pregnancy (primitive streak and early head fold stage) embryos including embryonic membranes and decidual tissue were processed for histological examination. From d 11 of gestation to birth embryos were processed and examined separately from embryonic membranes and uterine tissue.

Yolk sac carcinoma

Carcinomas were induced in the same strain of rats by grafting 7 d embryonic ectoderm obtained on d 8 of pregnancy (preprimitive streak stage) under the kidney capsule of adult male rats (Levak-Svajger et al. 1991) (Fig. 2). Embryos were treated with 0.5% trypsin (Worthington) and 2.5% pancreatin (Difco) in Tyrode's saline (calcium and magnesium free) for 15 min at 4 °C. Embryonic ectoderm was separated from the remainder of the conceptus with electrolytically sharpened tungsten needles and transferred with a micropipette under the kidney capsule of the 3-month-old males. Approximately 6 months after the operation 50% of animals developed a malignant

tumour accompanied by ascites. In addition to a primary tumour found on the kidney (spherical structures 0.5–6 cm in diameter) the entire peritoneal cavity was covered with metastatic nodules usually less than a couple of millimetres in diameter. A small (< 1 cm) piece of the primary tumour along with a few metastatic nodules were taken for immunohistochemistry from 5 different animals. A total of 10 malignant samples were processed for immunohistochemistry and each expressed a similar oncogene protein pattern.

Fixation, embedding and sectioning

The embryos and the tumour tissue were fixed overnight in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) at 4 °C. They were washed 3 times for 30 min in PBS followed by 30 min in 0.85% NaCl saline, then in an ethanol/saline mixture (1:1). The embryos and tissues were dehydrated in several steps up to 100% ethanol. The specimens were cleared in xylene, embedded in paraffin, and sectioned at 5 μm with a Reichert microtome. The sections were placed on Tespa coated microscope slides, deparaffinised with xylene twice for 15 min, washed with 100% ethanol twice for 5 min, 95% ethanol twice for 5 min, and 70% ethanol for 5 min. The samples were cleared in PBS and prepared for immunohistochemistry.

Immunohistochemistry

This was performed using the peroxidase-antiperoxidase complex (Pavelic et al. 1990). Endogenous peroxidase activity in tissue samples was blocked by incubation in methanol/3% H_2O_2 for 15 min. Slides were cleared in PBS and nonspecific binding was inhibited using normal rabbit serum (dilution 1:10 in PBS) in a humidity chamber for 30 min. Slides were blotted dry and the murine monoclonal antibodies (Molecular Oncology, Inc., Gaithersburg, MD, USA) diluted 1:100 in PBS were applied for 2 h at room temperature. The slides were washed 3 times in PBS containing 2% normal human serum. The secondary antibody (dilution 1:25 in PBS), rabbit immunoglobulins to mouse immunoglobulins (Dakopats, Denmark), was applied for 1 h at room temperatures. Finally, the peroxidase-antiperoxidase conjugate (dilution 1:100 in PBS) was applied for 12 h at room temperature. After washing for 10 min in PBS the colour reaction was initiated by using diaminobenzidine tetrahydrochloride as a chromogen.
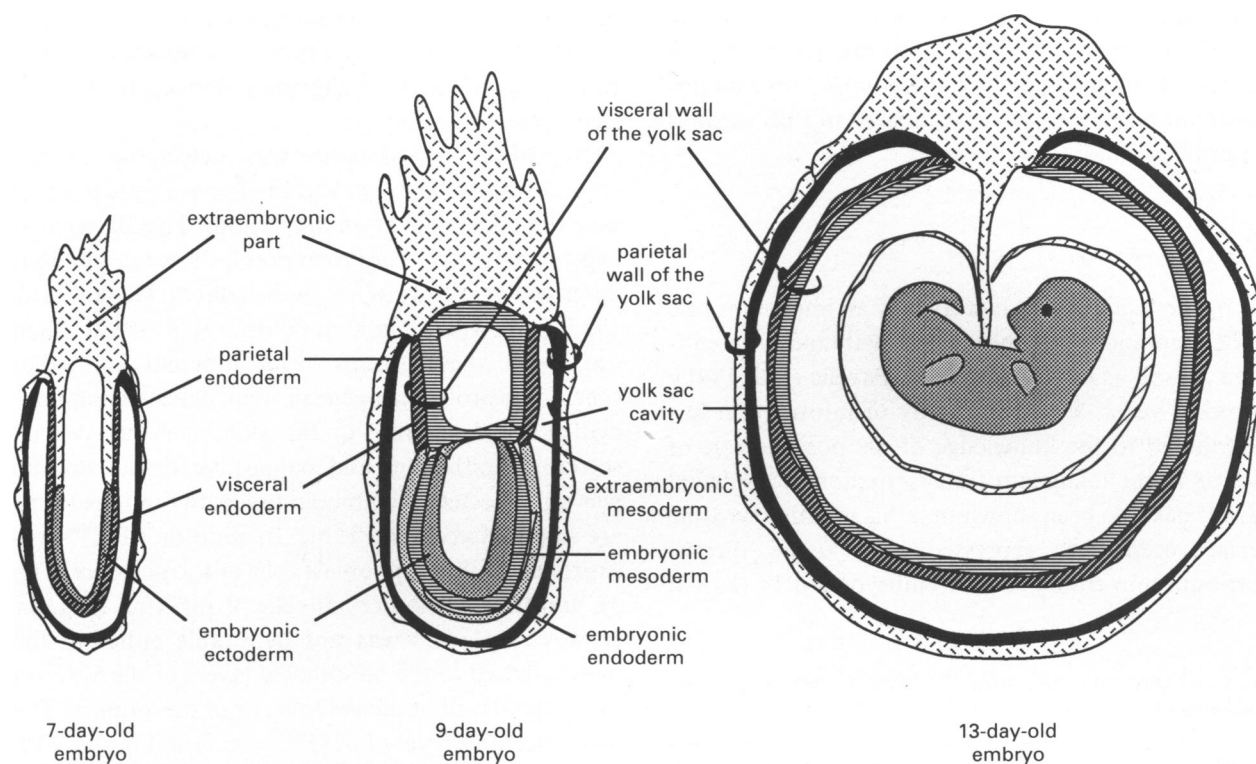
Fig. 1. Morphological relationship between conceptus and embryonic membranes during embryonic development. Note that progenitor cells of yolk sac endoderm are already present in 7 d embryo.
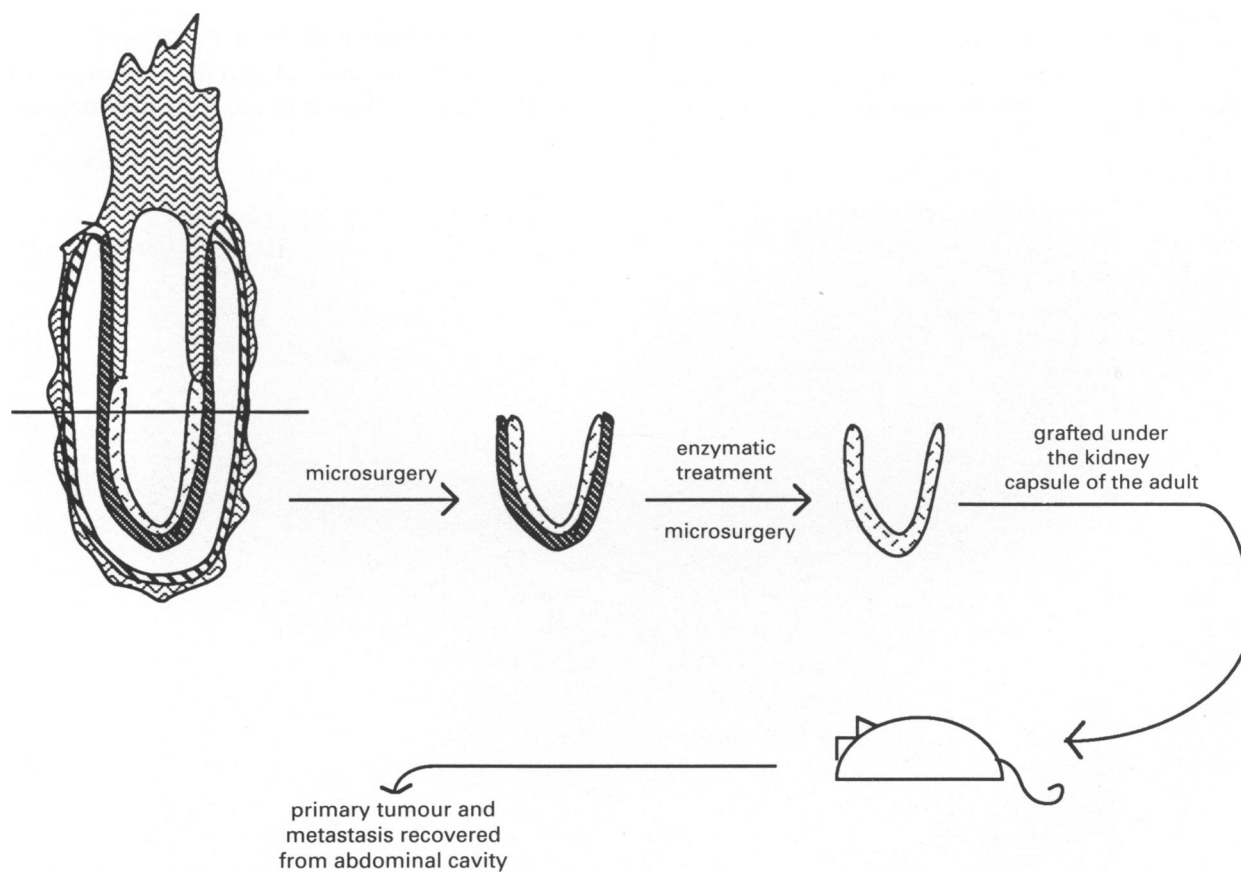

Fig. 2. Procedure used to obtain yolk sac carcinoma of rat.

As negative controls the tissues reacted with the primary antibody which had been preincubated with the peptide used to produce the c-erbB-2/neu monoclonal antibody and tissues incubated in PBS without the primary antibody were used.

RESULTS AND DISCUSSION

Oncogenes and growth factors play an important role in the regulation of embryonic growth and differentiation as well as in tumorigenesis (Pavelic et al. 1991). In recent years, a growing body of information has contributed to the knowledge of the possible role of p185^{neu} in the malignant transformation of epithelial cells. It has also been shown that this putative tyrosine kinase receptor is expressed in a tissue specific distribution in embryos after embryonic d 14 (Kokai

Table. *Expression of p185^{neu} during rat embryonic development*

Tissue	Day 12	Day 15	Day 20
Neural tissue	+	±	−
Connective tissue	+ +	+ +	+ +
Secretory epithelium	+ +	+ +	+ +
Squamous epithelium	−	−	−
Lymphoid tissue	−	−	−

Intensity of immunoreactivity: −, no staining; ±, positive and negative cells present; +, moderate staining; + +, strong staining.

et al. 1987). In this study we examined the expression of p185^{neu} in early embryos and extraembryonic tissues as well as in malignancy derived from these structures.

No p185^{neu} expression was detectable in the embryos before embryonic d 14. The *neu* gene product was first detected at midgestation. The pattern of expression seen was remarkably similar to that described in a study of *neu* in the fetal rat (Kokai et al. 1987) and c-erbB-2 in man (Quirke et al. 1989). As in our study, these groups, using different antibodies specific for proto-oncogene protein, detected immunocytochemical staining in the skin, nervous system, secretory epithelium and connective tissue. Protein was not detected in lymphoid tissue. Staining patterns are summarised in the Table. In addition p185^{neu} was expressed in the trophoblast cells of the placenta (Fig. 3), and in the endodermal cells of rat VYS (Fig. 4). However, p185^{neu} was not detectable either in the mesenchymal or the mesothelial layers of the VYS, in the parietal wall of the yolk sac, or in the amnion. The first detectable level of p185^{neu} was found in the VYS at d 11 of gestation and was present throughout the rest of pregnancy. The staining was confined to the periapical segment of the cytoplasm of endodermal cells (Fig. 4A). Towards the end of gestation the overall immunoreactivity was much stronger and dye deposits were prominent on the membranes of apically located endocytotic vesicles (Fig. 4B). The connective tissue and the mesothelium of the VYS were negative
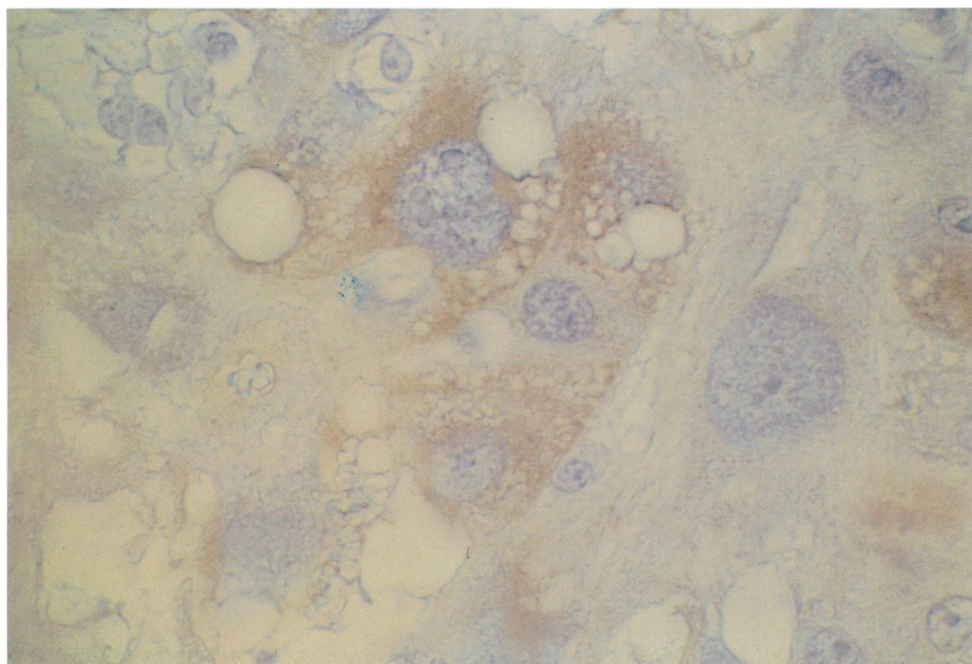

Fig. 3. Immunohistochemical localisation of p185^{neu} in the trophoblast cells of the placenta.
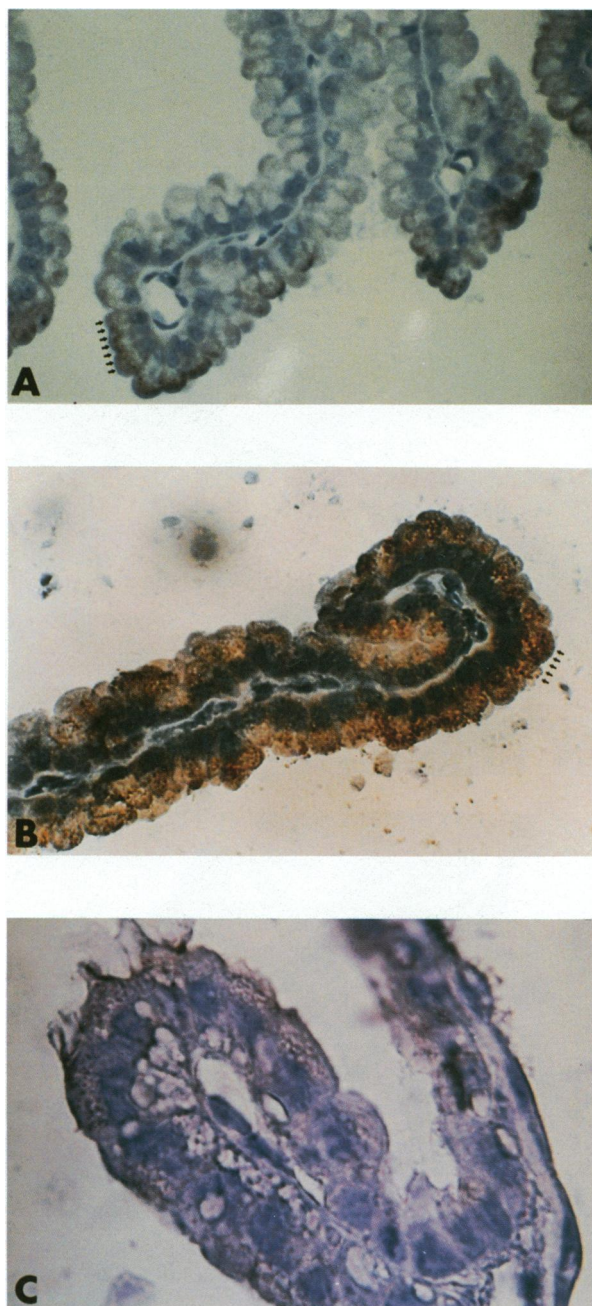
Fig. 4. Immunohistochemical localisation of p185^{neu} in the epithelium of the visceral yolk sac during d 12 (*A*) and d 17 (*B*) of gestation. The staining is confined to the periapical segment of the cytoplasm of endodermal cells (arrows). No staining is visible in the negative control (*C*).

for p185^{neu}, thus suggesting that this protein is expressed only in cells with a glandular function. We observed no difference in protein expression between the villous (placental part) and the smooth portion (located distally from placenta) of the VYS. The p185^{neu} expression appears at d 11 and continues to increase to peak levels just prior to delivery. It is possible that this observed increase is directly correlated with specific needs of the growing conceptus.

Even though the progenitor cells of the VYS endoderm can be traced from the stage where only 2 germ layers are present (early egg-cylinder stage) (Fig. 1) (Levak-Svajger et al. 1991) these cells show no sign of p185^{neu} expression (Fig. 5). The appearance of p185^{neu} in endodermal cells parallels the vascularisation of the VYS by the peripheral vitelline circulation, from which point on the VYS is able to perform a distinct glandular function (Lambson, 1966). We therefore believe that the expression of
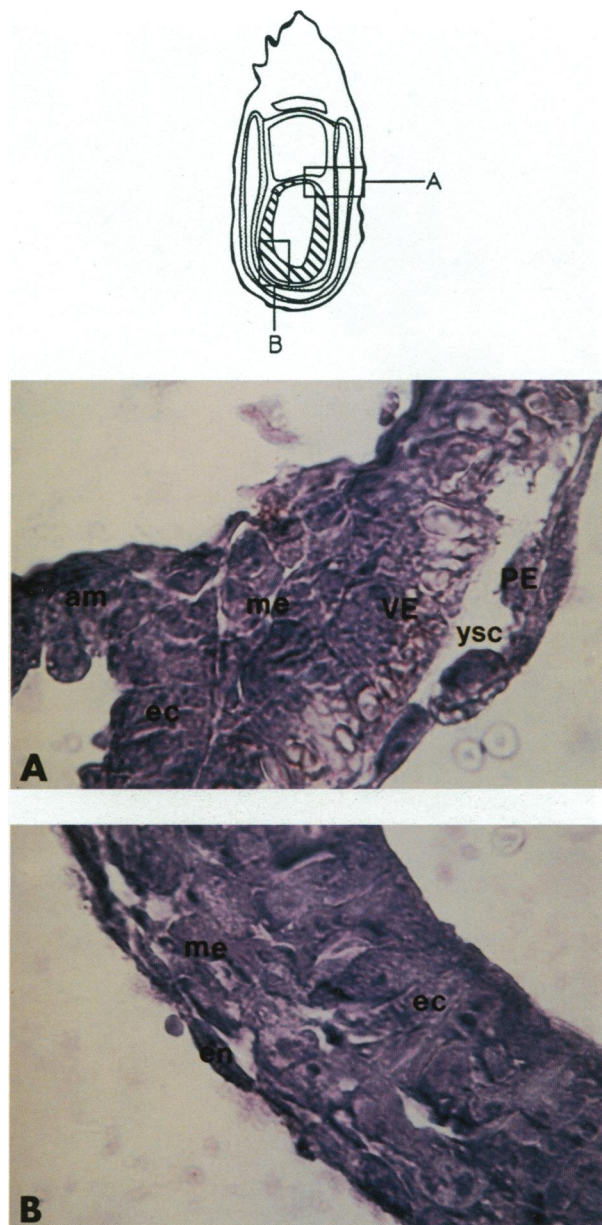
Fig. 5. Nine-day old embryo. No $p185^{neu}$ expression was observed in the embryo at this stage. am, amnion; ec, embryonic ectoderm; me, mesoderm; VE, visceral endoderm; PE, parietal endoderm; ysc, yolk sac cavity; en, embryonic endoderm.

$p185^{neu}$ can be directly correlated with the functional activity of these cells.

In the malignant counterpart of the rat yolk sac, yolk sac carcinoma, the *neu* product was observed only within columnar epithelial cells (the visceral component of the neoplasm), while parietal endoderm-like cells (which produce large amounts of basement membrane material) were not immuno-reactive (Fig. 6). This observation therefore provides additional evidence that the expression of $p185^{neu}$ is, even in the tumour tissue, restricted predominantly to the cells with a 'glandular-type' morphology (cylindrical shape, endocytotic vesicles). This is also suggested by the results of recent studies done by several groups using primary human tumours (McCann et al. 1990; Gusterson, 1992). They have reported over-expression of the *c-erbB*-2 oncoprotein in carcinomas of glandular epithelial origin (adenocarcinomas).

Considering the importance of the yolk sac placenta in rodent embryonic development along with the previously described role of all tyrosine kinases (including *neu*), it is reasonable to assume that this proto-oncogene product plays an important role in rat embryogenesis.
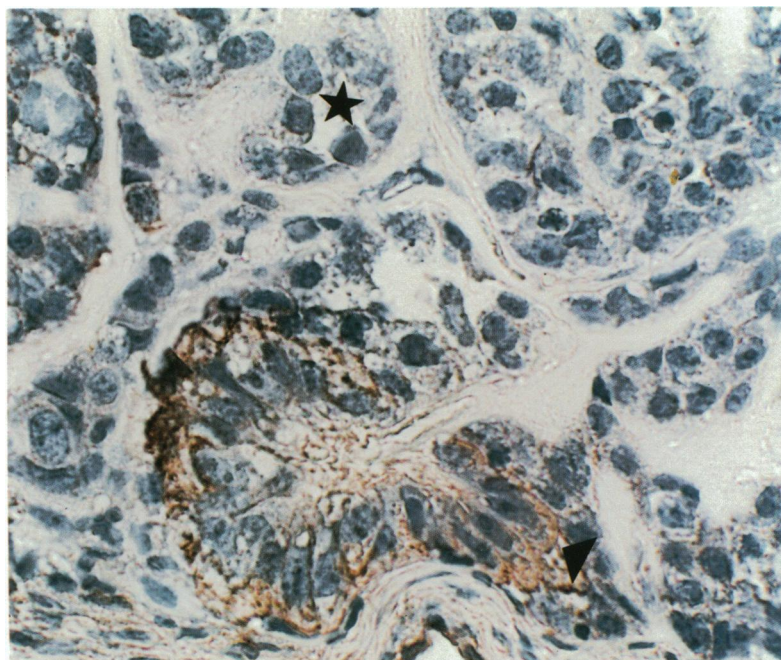
Fig. 6. Immunohistochemical localisation of p185^{neu} in the rat yolk sac carcinoma. The protein is localised only within the columnar visceral-like endoderm cells (arrowhead) and not in the parietal-like endoderm component of the neoplasm (asterisk).

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